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Effect of Lysine Residues Bound to Ornithine Side Chains of Gramicidin S on Its Antibacterial Activity

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Summary

Gramicidin S (GS) analogs, which have Lys residue extensions on the side chains of $Orn^{2,2'}$, were synthesized to study the effect of Lys chains on antibacterial activity and structure of GS. As Lys chains became longer, the analogs revealed reduced antibacterial activities against gram-positive bacteria, and the ring structure of GS distorted judging from circular dichroism (CD) spectra. No analogs were active against gram-negative bacteria. There was no correlation between the number of Lys residues and the leakage pattern of fluorescence dye from liposomes. Extension of Lys residues resulted in : 1) Distortion of the original structure of GS ; 2) Quantity-imbalance between hydrophilic (basic) and hydrophobic groups of GS analogs. Therefore, it is likely that the analogs do not bind properly to the membrane of gram-positive bacteria. It has been reported that analogs containing many basic amino acid residues are active against gram-negative bacteria. However, none were active in this study, suggesting that the proper distribution of positive charges (basic groups) as well as its quantity are important.

Key words : gramicidin S, antibacterial activity, structure, dye-leakage

Gramicidin S (GS, Fig. 1) is a peptide antibiotic produced by *Bacillus brevis*. GS has been traditionally reported to display antibacterial activity against gram-positive bacteria,[1] and moreover, recently reported to be active against gram-negative bacteria with solution-based assay by Kondejewski *et al*., showing its activity is media-dependent.[2] Many studies, using various GS-related compounds, have revealed the molecular features important for the activity of GS.[3] Nevertheless, functional mechanism of GS is still not well clarified.

It is remarkable that GS-related compounds containing extra basic amino acid residues were also active against gram-negative bacteria with conventional agar-based assay.[4] These findings have prompted us to study further the relationship between basicity and activity of GS to investigate its action mode on bacteria.

In this paper, we synthesized GS analogs shown in Fig. 1 and measured their antibacterial activities, CD spectra, and carboxyfluorescein (dye)-leakage ability. Based on these properties,

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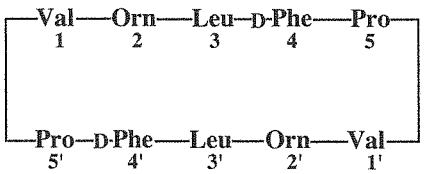
we discuss the relationship between basicity and activity of GS.

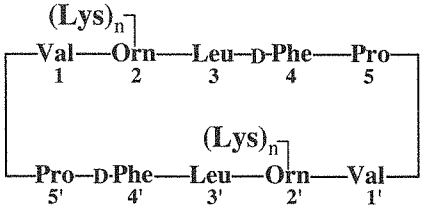
Materials and Methods

Peptide synthesis. Gramicidin S dihydrochloride and amino acid derivatives were products of SIGMA Chemical Company (St. Louis, U.S.A.) and Peptide Research Foundation (Minoo, Japan), respectively. Solvents and reagents used in this study were commercial products of high purity.

The synthesis followed the route in Fig. 2. Analog **4** was synthesized by coupling Boc-Lys(Z)-OH[5] onto the δ-amino groups of Orn residues of GS, using the mixed anhydride method.[6] Lys chains were elongated by repeating TFA cleavage of Boc groups and subsequent coupling of another protected Lys residue(s), yielding the analogs **5** and **6**. Final deprotection of peptides **4**, **5**, and **6** with HBr/AcOH gave the desired analogs **1**, **2**, and **3**, respectively.



Gramicidin S (GS)



Gramicidin S analogs (n=1, **1**; n=2, **2**; n=3, **3**)

Fig. 1 Structure of GS and its analogs.

All analogs were satisfactorily synthesized and their structures verified by elemental analyses, amino acid analyses, and nuclear magnetic resonace.

Antibacterial activity. The antibacterial activity assay was carried out according to the dilution method using Mueller Hinton agar (Difco) medium.[7] About 5 μl of the bacterial suspension (10^6 cells/ml) was inoculated with a multiple inoculator onto agar plates containing two-fold serial dilutions of each peptide. The plates were incubated at 37℃ for 18 h, and the minimum

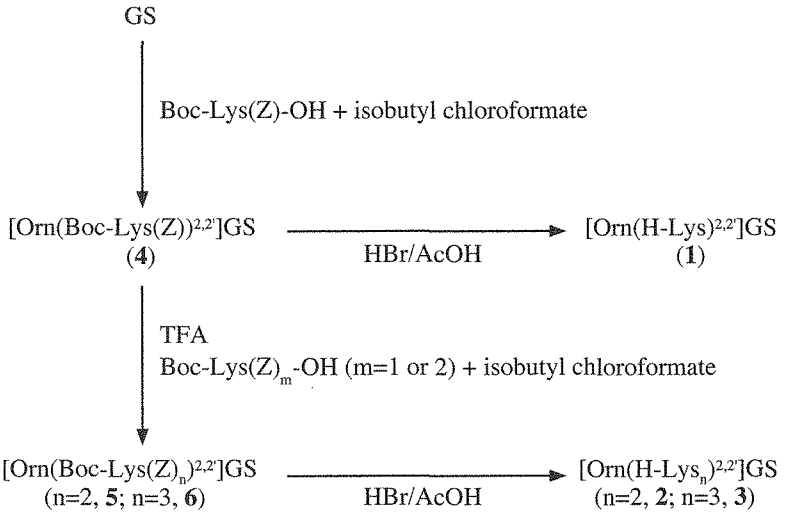


Fig. 2 Synthetic route of analogs.

concentration of the peptide, on which there was no visible growth, was noted.

CD measurements. CD spectra were recorded on a JASCO J-720 spectropolarimeter using quartz cells of 1 cm pathlength. Spectrum in the buffer was measured at a peptide concentration of 100 μM. Spectra in the presence of phospholipid liposomes were measured at a peptide concentration of 10 μM in 5 mM Tris-HCl buffer (pH 7.4). All the measurements were carried out at 25℃. The CD data were expressed as mean residue ellipticities.

Preparation of Phospholipid Liposomes. Dipalmytoyl phasphatidyl choline (DPPC) was a product of Ishizu Seiyaku Inc. (Osaka, Japan). DPPC was dissolved in chloroform and dried by breathing of nitrogen gas in a conical glass tube. The dried lipid was hydrated in 3 ml of 5 mM tris (hydroxymethyl) aminomethane (Tris)-HCl buffer (pH 7.4) with repeated vortex-mixing at 50℃ for 30 min. The suspension was sonicated at 50℃ for 20 min with BRANSON sonifier 250 and diluted to 25 ml with the same buffer. The mixture of uni- and multilamellar vesicles was used for the CD measurement without further purification. In the case of carboxyfluorescein -trapped unilamellar vesicles, the method described above was used except that the dried lipid (27 μmol) was hydrated in 2.0 ml of 0.1 M NaCl/5 mM 4-(2-hydroxyethyl)-1-piperizineethane sulfonic acid (HEPES) buffer (pH 7.4) containing 100 mM carboxyfluorescein. The mixture of carboxyfluorescein-trapped uni- and multilamellar vesicles was subjected to gel-filtration through a Bio-Gel A-15 m column (1.1 x 20 cm) in 0.1 M NaCl/5 mM HEPES buffer (pH 7.4). The separated small unilamellar vesicles were utilized in the carboxyfluorescein leakage measurements.

Carboxyfluorescein (dye) Leakage. 5(6)-Carboxyfluorescein was a product of Ishizu Seiyaku Inc. Fluorescence intensity was recorded on a Hitachi F-4000 fluorescence spectrophotometer at 26℃. Carboxyfluorescein experiment was carried out according to the method of Utsumi *et al.*[8] To 2 ml of 0.1 M NaCl/5 mM HEPES buffer (pH 7.4) in a cuvette was added 50 μl of the liposome containing 100 mM carboxyfluorescein and appropriate dilution of the analogs. Five minutes later the fluorescence intensity was measured. To determine the fluorescence intensity derived from 100% dye-release, 10 μl of Triton X-100 solution (20% in phosphate buffer) was added to cause lysis of the liposomes. The percentage of the dye-release caused by peptides was evaluated by the following equation, 100(F-Fo)/(Ft-Fo), where F is the fluorescence intensity achieved by peptides, Fo and Ft are intensities without peptides and of post-Triton X-100 treatment, respectively.

Results and Discussion

Antibacterial activities of the analogs against some bacteria are summarized in Table. Analogs **1**, **2**, and **3** showed less activity against gram-positive bacteria in comparison with GS, and no activity in some cases. Interestingly, however, among these analogs, analog **1** was the most active, and the activity diminished as the Lys chains elongated. This suggests that the longer chains reduce the antibacterial activity against gram-positive bacteria by some mean. In contrast, analogs **1**, **2**, and **3** showed no considerable activities against gram-negative bacteria, although they contained many basic amino acid residues. This suggests that another factor, other than quantity of basicity, is important for the activity.

CD spectra, taken in MeOH or in Tris-HCl buffer (pH 7.4) with or without DPPC liposome,

Table Antibacterial Activity of GS and its Analogs

organism	MIC[a] [μg/ml]			
	GS	**1**	**2**	**3**
gram-positive				
B. subtilis PCI 219	6.25	12.5	12.5	25
S. aureus FEA 209P	3.13	12.5	50	100
S. pneumoniae Type-III	12.5	100	>100	>100
gram-negative				
E. coli NIHJ JC-2	>100	>100	>100	>100
P. vulgaris IFO 3988	>100	>100	>100	>100
P. aeruginosa U-31	>100	>100	>100	>100

a) Minimum inhibitory concentration.

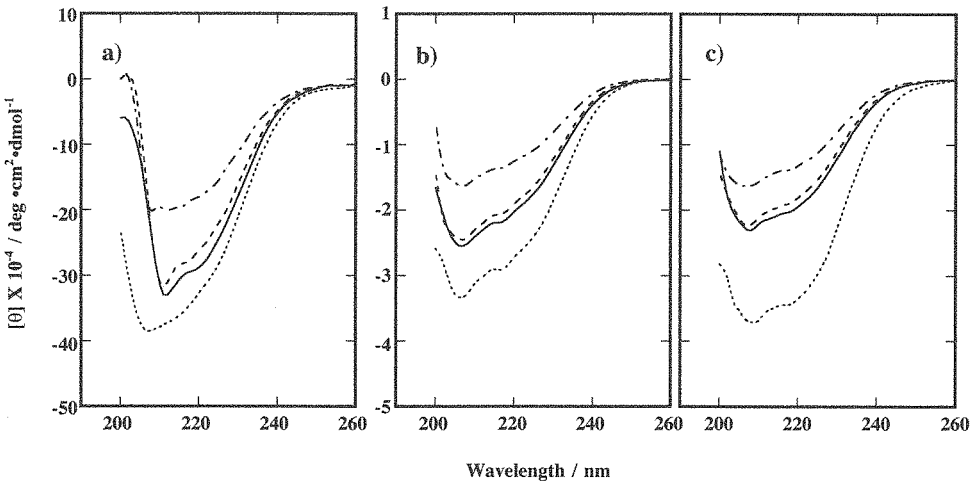


Fig. 3 CD spectra GS and analogs (**1**, **2**, and **3**).
···GS ; —**1** ; --- **2** ; and -·- **3**. a) In MeOH. b) In Tris-HCl buffer (pH 7.4) without DPPC liposomes. c) In Tris-HCl buffer (pH 7.4) with DPPC liposomes.

are shown in Fig. 3. GS and the analogs had double minima in the range of 200-220 nm. In any case the troughs became shallower with the increase of Lys residues. Lee *et al.* reported that double minimum band of GS is attributable to its characteristic structures, β-sheet and β-turn.[9] Therefore, Lys chains possibly distorted the original structure of GS to some extent.

Dye-leakage from DPPC liposomes were measured to compare the membrane perturbation ability of analogs **1**, **2**, and **3** with that of GS (Fig. 4). Analog **1** and GS caused their maximum leakage in a wide range (20-80 μM). It is interesting that the former reached 100% leakage but the latter only 80%. The leakage-activity of analog **2** was proportional to concentration, and stronger than that of GS in the high concentration range tested (60-70 μM). It is noteworthy that analog **3** showed the full leakage in the relatively wide range (40-80 μM). There is no clear correlation between antibacterial activity and dye-leakage activity, reflecting the different nature of biomembrane and liposome membrane.

It has been reported that the amphiphilic nature of GS is responsible for interaction with

membrane and subsequent events.[3] Interestingly, the analogs with longer Lys chains had shorter retention time on C_{18} reversed-phase column (data not shown) and showed the weaker activity against gram-positive bacteria. Moreover, Shimohigashi *et al.* suggested that the hydrophobic and hydrophilic groups have intrinsic roles in interaction with various biomembranes, using GS analogs containing α, β-dehydro-Phe residues.[10] From this viewpoint, long Lys chains seem to cause imbalance between hydrophobicity and hydrophilicity and interfere with the normal interaction of GS. Sakamoto *et al.* reported that GS should hold the active conformation for the antibacterial activity.[11] Therefore, the analogs in this study have lost the active conformation, judging from CD spectra, due to the electrostatic repulsion and/or steric hindrance among the Lys chains. It is likely that the effects described above cooperatively caused the reduction of activity against gram-positive bacteria.

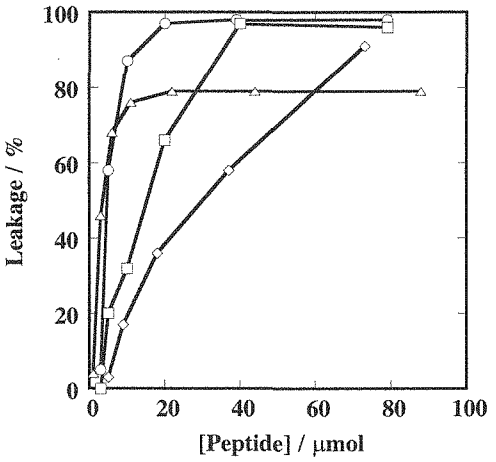


Fig. 4 Profiles of carboxyfluorescein leakage from DPPC by GS and analogs **1**, **2**, and **3**. -△- GS ; -○- **1** ; -◇- **2** ; -□- **3**.

On the other hand, analogs **1**, **2**, and **3** displayed no activity against gram-negative bacteria. GS-related compounds synthesized by Ando *et al.*[12] have the basic residues introduced into a cyclic structure, whereas analogs in this study have Lys residues on the side chains. Comparing their structures, there seems to be the essential factor for the antibacterial activity against gram-negative bacteria, *i.e.*, the proper distribution of positive charges as well as the proper quantity-balance between hydrophobic and hydrophilic groups. This is consistent with the conclusion of Ando *et al.*[12]

Our findings may be helpful in further understanding of the mode of action and the essential factors for the activity of GS. These in turn can lead to systematic development of therapeutically useful GS analogs with desired properties. Study on balance between hydrophobicity and hydrophilicity in GS analogs is now in progress in our laboratory using *de novo* designed analogs.

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グラミシジンSのオルニチン側鎖に結合したリジン残基の抗菌活性に対する影響について

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摘　　要

2,2'位オルニチン残基の側鎖アミノ基に1－3個のリジン残基を結合したグラミシジンS（GS）アナログを合成し，活性と構造に対する影響を検討した．

GSに結合したリジン残基の個数が増えるにつれて，グラム陽性菌に対する抗菌活性は低下したが，グラム陰性菌に対してはどのアナログも活性がなかった．また円偏光二色性（CD）スペクトルから，リジン残基の増加につれて，GS本来の環構造が歪んでいくことがわかった．リジン残基数とリポソームからの蛍光色素漏出パターンに相関は見られなかった．

リジン残基を延長することによって，1）GSの構造が崩れる，2）親水性（塩基性）と疎水性のバランスが崩れる，ことが示唆された．その結果，GSと膜との正常な結合が妨げられ，グラム陽性菌に対する活性の低下が起こる，と考えられた．

グラム陰性菌に抗菌活性があると報告されているペプチドは，塩基性残基を多く含んでいる．しかし，今回どのアナログも効果がなかったことより，グラム陰性菌に対して活性を示すためには，親水性基（塩基性基）と疎水性基の数のバランスだけではなく，塩基性基の適当な配置も必要である，と考えられた．